

DESCRIPTION

METHOD FOR MEASUREMENT OF HEPATITIS C VIRUS

Field of the Invention

5 The present invention relates to a method for
detection of the hepatitis C virus (HCV), and more
specifically it relates to a method for measurement of
HCV core antigen or for simultaneous measurement of HCV
core antigen and HCV core antibodies. The method is
10 particularly effective for screening of multiple blood
samples and the like.

Background Art

Hepatitis caused by infection with HCV (hepatitis C
virus) becomes chronic with high incidence, and as the
15 infection period is prolonged it often progresses to
liver cirrhosis and hepatocellular carcinoma. However,
since infection with HCV occurs mainly through blood and
blood-derived components, it is possible to identify and
eliminate the source of infection to block the infection
20 route. Current methods of identifying infection sources
are primarily methods of detecting antibodies against HCV
polypeptides, but methods are being sought that can
identify infection sources with greater accuracy.

Such methods are being sought because of the
25 existence of a period of time known as the "window
period" after HCV infection during which the antigen is
present but antibodies are not yet produced. Antibody
testing cannot determine whether serum taken during this
period is infected or not. Therefore, there is a risk of
30 secondary infection by the blood derived components, such
as blood donation, blood components, factors from blood,
contaminated specimens in the window period, because
blood donor is screened by the antibody test that can not
exclude such specimens. For this reason it has been
35 necessary to detect HCV itself, that is, HCV particles,
instead of antibodies against HCV polypeptides to reduce
the risk.

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Detection and measurement of HCV itself is possible by detecting antigens or genome (RNA) in the HCV particles. Here, an antigen in the HCV particles could be core antigen or an envelope antigen (E1, E2).

5 A lot of variants were reported in the antigenic region of envelope protein such as hyper variable region. In addition, heterogeneities of sequences between genotypes were reported. In order to detect all of these variants and heterogeneous sequences, it is necessary to
10 use probes that bind to several regions respectively.

Here, "probe" will be used to refer to a molecule that binds specifically to an antigen, for example a molecule which recognizes and binds to an antigen molecule, such as a receptor, antibody, recombinant
15 antibody, functional molecule or functional structure.

The amino acid sequences of core antigen were more conserved than those of envelope antigens. By selecting well conserved regions among several HCV genotypes, a probe recognizing core antigen of all genotype could be
20 obtained. Consequently, the method whose results should not be affected by genotypes will be constructed.

However, one point must be considered in constructing systems for detection of antigens. Specifically, it is highly possible that antibodies in
25 specimens from the subject compete with the antigen-detecting probe for the binding sites, resulting in lowering the detection of sensitivity for the antigen by interfering probe binding. A method would be constructed by using probes recognizing the regions that could not be
30 bound or interfered by antibodies in the specimens. However, it is difficult to prepare probes that fulfill these conditions for molecules reported to have multiple antibody-binding sites, such as HCV core antigen.

Thus, detection of antigen molecules requires
35 elimination of antibodies that inhibit probe binding. Methods of elimination include methods of elimination based on physical principles, for example methods

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utilizing differences in molecular weight for separation and fractionation of HCV particles and antibodies. Examples of such methods include gel filtration, ultracentrifugation, density gradient centrifugation and molecular weight fractionation using membranes such as ultrafiltration membranes. However, since antibodies often form complexes with other biomolecules whereby they become high molecular weight entities, their separation from HCV particles is difficult by methods based on physical principles. These methods also employ special equipment during the processing steps, which makes their application difficult for mass screening, such as blood screening.

HCV particles are preferentially precipitated by the difference of their solubility in water-based solution containing PEG (polyethylene glycol), which alter the microenvironment of water. However, it will be very difficult to separate antibodies and their complex with antigens from HCV particles, because these components precipitated in same fractions. Moreover, HCV particles often form immune complexes between the antigens in the HCV particles and antibodies that recognize them, and it is difficult to separate only the antibodies or antigens from the immune complexes.

The methods implemented are therefore ones whereby substances (antibodies, etc.) that inhibit probe functions are eliminated by destroying their functions. One such method for losing antibody functions is a method in which the antibody protein is denatured by exposure to conditions that denature the protein structure, but it is essential here to destroy the function of the antibody while not eliminating the function of the object antigen, i.e. the function of binding with the probe, which means not losing the epitope or allowing the epitope to be displayed again, if the probe is an antibody.

The target function of a method of determining the HCV infection will differ depending on the goal.

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Antibody testing is a method only to determine the specimen containing antibodies against HCV. When antibodies against HCV are present in a specimen, there are cases where the specimen donor is currently contains HCV because of active injection of HCV, while there are other cases where the specimen does not contain HCV because of HCV elimination from body by treatment or material recovery it is difficult to discriminate these therefore cases based on the presence or absence of antibodies.

The important function of antigen test is to determine whether or not HCV is present in a specimen or to indicate the level of HCV when it is present. It is not dependent on the question of whether or not antibodies are present.

For treatment, HCV antibody testing provides important information for determining whether HCV is the main cause of hepatitis. However, the test for HCV itself is require for definite diagnosis. Determining whether HCV has been eliminated from the body is important in judging the efficacy of treatment. The information of the level of antigen is essential in making a such decision for treatment. That is, for treatment it is important to know whether or not and at what level the antigen is present, regardless of the presence or absence of antibodies. For treatment, then, the most important testing methods are those which indicate the presence or absence and the level of antigen.

For blood and blood-derived components, preventing secondary infection is of greatest importance. For this purpose, testing methods that assess the risk of HCV infection. Antibody testing is currently being used as the primary testing method in this field.

However, as explained above, antibody testing cannot determine whether serum is infected during the window period after HCV infection. Consequently, when blood-

derived substances such as transfusion and blood components, blood preparations, etc. are utilized for screening by antibody testing, there is a risk of secondary infection by specimens in the window period.

5 Coupling this with antigen testing is desired in order to reduce this risk, but antigen testing is still not implemented for mass screening for blood donation.

10 If a testing method existed that could determine the presence or absence of antigen with a theoretical accuracy (sensitivity, specificity) of 100%, that method could be used as the sole testing method. However any method have a limit of the detection sensitivity and cannot measure levels below that detection sensitivity. Thus, no testing method exists that can discriminate with
15 100% accuracy. There remains the possibility of missing the source of infection by antigen testing alone, and it is for this reason that measurement of both antibody and antigen is necessary to reduce the risk of secondary infection. If the antigen test, having high sensitivity and specificity sufficient for mass screening, is
20 available, both antigen and antibody test were required for the screening. This requirement will result in higher cost of the screening, because the number of tests performed with samples should increase than present.

25 It is therefore clear that if measurement of antigen and antibody by the same method becomes possible, it will allow a reduction in the number of tests performed in the field, thus providing a major effect.

30 As already mentioned, despite the development of antibody detecting methods and antigen detecting methods, when it is attempted to detect antigen under conditions for detecting antibodies as alluded to above, the antigen cannot be efficiently detected because of the presence of antibodies which inhibit binding of probes that detect
35 the antigen. Even under conditions for detection of antigen, however, the adopted methods eliminate antibodies that compete against detection of antigen, as

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explained above, and therefore antibodies cannot be detected. The currently reported methods, therefore, do not allow detection of antigen and antibody by a single method.

5 Disclosure of the Invention

For the purpose of reducing secondary infection when utilizing blood and materials derived from blood, it is not necessary to distinguish between infected persons and formerly infected persons, as it is sufficient to be able to determine whether or not antibody or antigen is present. The present invention therefore provides a method of detecting antigen in specimens during periods in which they contain no antibodies, such as during window periods, and detecting the antigen or the antigen and antibodies in specimens during periods in which the antibodies are present; it therefore provides a new testing method which is desirable for testing of blood and blood-derived substances.

In order to solve the problems mentioned above, the present invention provides a method for measurement of the hepatitis C virus (HCV), characterized by measuring HCV core antigen by its binding with one or more probes in the presence of one or more detergents with one or more alkyl chains and one or more secondary to quaternary amines or one or more non-ionic detergents, or both.

The present invention further provides a method for measurement of HCV core antigen by the aforementioned method while also measuring HCV core antibody by its binding with a probe.

30 BRIEF EXPLANATION OF DRAWINGS

Fig. 1 is a graph showing a comparison of antibody titer of monoclonal antibody C11-15 with antibody titers of other monoclonal antibodies C11-3, C11-7, C11-10 and C11-14.

35 Fig. 2 is a graph showing a result of ELISA for measuring HCV-RNA positive specimen using as primary antigen immobilized on a solid phase each of various

monoclonal antibodies alone, or mixture thereof.

Embodiments for Carrying out the Invention

5 The method of detecting HCV infection provided by the present invention is a method whereby the antigen is detected in a specimen during a period in which no antibodies are present, such as during a window period, and whereby the antigen or both the antibody and antigen are detected during periods in which antibodies are present. In other words, during window periods in which
10 no antibodies are present, the fact that no antibodies are present means that there is no need to eliminate antibodies during detection of the antigen. Consequently, pre-processing for detection of the antigen is no longer necessary.

15 Nevertheless, the regions on the antigen, that are recognized by the probe, must be displayed for detection, because the core antigens are packaged into virus particle. HCV particles are believed to have a structure wherein complexes are formed between the genomic RNA and the core antigen, forming particles which are coated with
20 lipid outer membranes comprising envelope proteins. They are also believed to exist in the blood in the form of complexes with low density lipoproteins (LDLs) and or anti-HCV antibodies. The probe cannot recognize and bind with the core antigen when it is in the viral particles present in the blood. Thus, detection of core antigen requires processing to remove the structure enveloping the core antigen to allow recognition of the core antigen by the probe.

25 The present invention therefore further provides a reaction method comprising reaction conditions and a reaction system by which core antigen in HCV particles contained in a specimen is displayed so that it can be recognized by a probe for recognition of the core
30 antigen, as well as a reagent that includes such a reaction system.

During periods in which antibodies are present at a

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sufficient level, antibodies against the core antigen that compete for the binding site of the probe are sometimes present in the specimen, and this can lower the detection sensitivity for the core antigen. Also, when the core antigen is exposed to allow the probe to bind in the presence of the antibodies to compete with the probe for the binding, the levels of antibodies that bind the antigen for the antibody test will be reduced by their absorption to the displayed core antigen of the virus particles. As a result, the sensitivity of the antibody test, which measure the amount of immuno-complex of antibodies with the antigens for the antibody test.

Consequently, while the antigen used for detection of HCV antibodies may be one consisting solely of the core antigen epitope, it is preferably a peptide or polypeptide including an HCV epitope other than the core antigen. It may also be a peptide or polypeptide, or compound, other than the peptide or polypeptide including the HCV epitope, which mimics the HCV epitope.

However, the probe used for detection of the core antigen and the HCV epitope or the compound substituting for the HCV epitope are preferably not entities that bind through mutual recognition.

The antibody used as the probe for the HCV core antigen or the labeled antibody for detection of the HCV core antigen may be a polyclonal antibody obtained by immunization of an experimental animal such as a mouse, rabbit, chicken, goat, sheep, cow, etc.; a monoclonal antibody produced by a hybridoma obtained by separating spleen cells from an immunized individual and fusing them with myeloma cells; a monoclonal antibody produced by spleen cells or blood leukocytes that have been immortalized with the EB virus; or a monoclonal antibody produced by a human or chimpanzee infected with HCV; a recombinant antibody produced by cells transformed by a recombinant antibody gene constructed by combining an immunoglobulin constant region gene fragment with a

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variable region gene fragment obtained from immunoglobulin cDNA or chromosomal DNA from a mouse, human, etc., a variable region gene fragment constructed by combining a portion of immunoglobulin cDNA or
5 chromosomal DNA with an artificially created sequence, a variable region gene fragment constructed using an artificial gene sequence or a variable region gene fragment created by a gene recombination method using these as materials; a phage antibody created by fusing
10 any of the aforementioned variable region gene fragments with the structural protein of a bacteriophage, for example; or a recombinant antibody produced by cells transformed by a recombinant antibody gene constructed by combining any of the aforementioned variable region gene
15 fragments with another appropriate gene fragment, such as a portion of the myc gene.

Probes produced by artificial introduction of variable regions into trypsin molecules, probes obtained by artificially modifying molecules that bind
20 specifically to proteins such as receptors, and other probes prepared by combinatorial chemistry techniques, may be used so long as they exhibit high specificity and affinity for the core antigen.

The above-mentioned monoclonal antibodies can be
25 easily prepared by those skilled in the art. Preparation of monoclonal antibodies from hybridomas is well-known. An example thereof is periodic immunization of BALB/c mice or the like either intraperitoneally or intradermally using the fused polypeptide or polypeptide
30 (hereunder referred to as "the present antigen") as an antigen either alone or bound to BSA, KLH or the like, in simple form or in admixture with an adjuvant such as Freund's complete adjuvant. The present antigen is administered through the caudal vein as a booster
35 immunization once the blood antibody titer has increased, and after antiseptic extraction of the spleen, cell fusion is carried out with an appropriate mouse myeloma

cell line to obtain hybridomas. This method can be carried out according to the method of Kohler and Milstein (Nature 256: 495-497, 1975).

5 The hybridoma cell lines obtained by the above procedure are cultured in a suitable culture solution, and then a hybridoma cell line producing antibodies that exhibit specific reaction with the present antigen is selected and cloned. The cloning of the antibody-producing hybridoma may be accomplished by the limiting
10 dilution method, or the soft agar method (Eur. J. Immunol. 6: 511-519, 1976). The monoclonal antibody produced is purified by a method such as column chromatography using Protein A, etc.

15 A molecule to be used as a probe may be prepared instead of the aforementioned monoclonal antibody. For example, recombinant antibodies are discussed in detail in a general outline by Hoogenboon (Trends in Biotechnology, 15: 62-70, 1997).

20 According to the invention, the antigen used as a probe for HCV core antibodies in a specimen or the antigen used for production of the HCV core antibodies is, specifically, a polypeptide having an amino acid sequence represented for example by SEQ.ID. No.1 or No.2, or a fused polypeptide including one of the amino acid
25 sequences listed as SEQ.ID. Nos.3 to 6, and these can be obtained by recombinant expression of DNA coding therefor.

30 The principle for detection in this case may be a method commonly used for immunoassay, such as the enzyme-labeled antibody method, fluorescent labeling method, radioisotope labeling method, etc., and the principle for enzyme detection in the enzyme-labeled antibody method is the colorimetric method, fluorescent method, chemiluminescence method, etc. The method used for
35 detection of the antibody may be one which is commonly employed for antibody detection, such as the double-antigen sandwich method, and a one-step sandwich system

may likewise be used for detection of the antigen as well.

One mode of the invention is the reaction system described below. (1) A probe for the HCV core antigen, for example antibodies for the HCV core antigen, and (2) a compound comprising the HCV epitopes, for example a peptides, peptide compounds or polypeptides comprising the HCV epitopes, or a mixture thereof, are immobilized on a carrier used for immunoassay, such as a microtiter plate. In order to capture core antigens and HCV antibodies in the specimen, the immobilized carrier is reacted with the specimens in the solution that contains the compounds to expose the core antigen in the virus particles or complexes with the particles and not to inhibit the function of HCV antibodies for binding to the HCV epitopes.

After then removing the unbound components in the specimen by, for example, washing the carrier with a suitable buffer solution, it is reacted with a reaction solution containing probes that recognizes the core antigen bound to the carrier, for example an enzyme-labeled antibodies for the core antigen, and probes that recognizes an antibody for the HCV epitope bound to the carrier, for example an enzyme-labeled anti-human antibody mouse monoclonal antibody, to cause specific binding with the core antigen and the antibodies to the HCV epitopes captured the carrier. After the reaction, the carrier is washed with a suitable buffer solution to remove the unreacted components, and detection of the label by an appropriate method will allow detection of the core antigen and the antibodies to the HCV epitope, which are present in the specimen.

It will be readily apparent to researchers in the relevant field that this may be applied as well to B/F separation methods that can be used for common immunoassay methods, such as immunochromatography.

Reaction conditions suitable for antigen detection

5 The reaction system suitable for antigen detection in the system provided by the present invention is a system under mild conditions which will not destroy the function of the antibody to the HCV epitopes, while also being under conditions that will sufficiently expose the region recognized by the antibody probe which is to recognize the HCV antigen amidst the HCV particles that are exist as a complex structure in the specimen.

10 It has been demonstrated that core antigen can be detected if the viral particles that have been separated by ultracentrifugation (Takahashi et al., 1996, J. Gen. Virol., 73:667-672) or HCV particles which have been aggregated and precipitated with polyethylene glycol are
15 treated with a non-ionic detergent such as Tween80 or Triton X100 (Kashiwakuma et al., 1996, J. Immunological Methods 190:79-89); in the former case, however, the detection sensitivity is insufficient and it is doubtful whether sufficient antigen is exposed. In the latter
20 case, treatment agents other than detergents were added to inactivate the antibodies. In addition nothing is mentioned about the actual effect of the detergent.

For the present invention, the conditions were first studied on the basis of the detergent, and by preparing
25 reaction solutions with detergent-based compositions, and it has become possible to efficiently detect antigen in HCV particles by simply diluting the specimen with the reaction solution without applying pre-processing involving procedures such as centrifugation and heating
30 as in hitherto reported HCV antigen detection systems.

In order to construct the system which this invention provide, it is necessary to effectively extract
the core antigen from the viral particles, suppress
interaction between the various substances in the serum,
35 and provide conditions which allow efficient reaction between the probe and the antigen. As detergents which are effective for this purpose there may be mentioned

detergents with alkyl groups and secondary to quaternary amines in the same molecule, and non-ionic detergents.

5 In a detergents comprising with alkyl chains and secondary to quaternary amines, the alkyl chain is preferably a linear alkyl chain, with the number of carbon atoms being preferably 10 or greater and more preferably 12-16. The amine is preferably a tertiary amine or quaternary amine (ammonium). As specific detergents there may be mentioned dodecyl-N-sarcosinic
10 acid, dodecyltrimethylammonium salts, cetyltrimethylammonium salts, 3-(dodecyldimethylammonio)-1-propanesulfonic acid, 3-(tetradecyldimethylammonio)-1-propanesulfonic acid, dodecylpyrimidium salts, cetylpyrimidium salts, decanoyl-N-methylglucamide (MEGA-
15 10), dodecyl-N-betaine, and the like. Dodecyl-N-sarcosinic acid and dodecyltrimethylammonium salts are preferred.

The aforementioned non-ionic detergent is preferably one with a hydrophilic-lipophilic balance of 12 to 14,
20 with polyoxyethylene isooctyl phenyl ethers such as Triton X100, Triton X114, etc. and polyoxyethylene nonyl phenyl ethers such as Nonidet P40, Triton N101, Nikkol NP, etc. being preferred.

According to the invention the above-mentioned two
25 types of detergents may be used alone, but their combinations are more preferable since synergistic effects can be achieved.

The present inventors found that a carrier, which was immobilized antigens containing HCV epitopes for
30 detection of HCV antibodies and antibodies to HCV antigens for the detection of HCV antigen, is able to efficiently capture HCV antigen, HCV antibodies in the specimens without HCV antibodies, or HCV antigen, respectively. The inventors also found that the carrier
35 efficiently capture both HCV antigens and antibodies in the specimens containing HCV antigens and antibodies, and gave higher signals derived by their binding. Based upon

these findings, the inventors have completed the present invention.

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5 A method for simultaneously detecting an viral antigen and antibodies to virus related portion has been already reported for HIV (Weber et al., J. Clinic. Microbiol., 36: 2235-2239, 1998). In the case of HIV, it is effective to detect p24 which is gag protein for a viral antigen test. On the other hand, for test for an antibody against viral antigen, it is effective to detect antibodies against envelop protein and p19 which is a gag protein. Therefore, a method for simultaneously detecting a viral antigen and an antibody against viral antigen is established by combining an antigen test which detects g24 that is a gag protein, and an antibody test which detects antibodies against envelop protein and p19 that is a part of gag protein.

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35 In such a case wherein epitopes used for detection of viral antigens is different from epitopes recognized by antibodies in a specimen used for detection of viral antibodies, it is relatively easy to construct a method for simultaneously detecting viral antigens and an antibodies against viral antigens. This is because, for example, in the case of HIV test, the antigen p24 recognized by a probe for detection of the antigen, for example, a monoclonal antibody against HIV epitope, and antigens recognized by antibodies contained in a specimen from a patient, which are envelop protein and p19 that is a part of the gag protein are different proteins, and therefore the probe used for the antigen test cannot recognize the envelop protein and the p19 which is a part of the gag protein. Accordingly, there is no interference between the antigen detection system and the antibody detection system, for example a non-specific reaction, decrease in sensitivity caused by competitive reaction between the probe and HIV epitope used for antibody detection.

However, for detection of antibodies against HCV

epitopes, the detection of antibodies against core antigen is highly useful from a clinical point of view (Chiba et al., Proc. Natl. Acad. Sci. USA 88: 4641-4645, 1991; Bresters et al., Vox Sang, 62: 213-217, 1992).

5 Therefore, it is essential for the antibody detection to detect an antibodies against the core antigen epitopes. On the other hand, for the antigen detection, detection of the core antigen is most useful in detection of HCV antigens, because, of antigens which form viral
10 particles, the mutation rate of the core antigen is lower than that of other antigens such as E1 and E2. Namely, to construct an assay system which simultaneously detects HCV antigen and HCV antibodies, the same antigen, i.e.,
15 the core antigen must be used for both the antigen detection system and the antibody detection system.

Therefore, if the core antigen is used without any improvement, the following problems are caused: a monoclonal antibody against the core antigen used for the detection of antigen binds to the core antigen used for
20 the detection of antibody, resulting in decrease in sensitivity for detection of core antigen in a specimen; the monoclonal antibody binds to the antigen used for the detections of antibody resulting in nonspecific reaction in the antigen test; and the HCV epitope for the antibody
25 test is masked resulting in decrease in sensitivity.

To solve the above-mentioned problems, the present inventors found that both the antigen and antibodies can be efficiently detected simultaneously (by distinguishing the epitopes recognized by monoclonal antibody in the
30 antigen detection from the epitopes of the core antigen for antibodies present in the specimen) and completed the present invention.

An combination of epitopes suitable for simultaneous detection of the antigen and antibodies is shown in
35 detail in Examples described hereinafter.

Regarding the epitopes for antibodies in a specimen against the core antigen, various epitope analyses show

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that the most important regions are present at the N-terminal region of the core antigen, especially from the position 1 to the position 40 of the HCV polypeptide (Okamoto et al., Hepatology 15: 180-186, 1992; Sallsberg et al. J. Clinical. Microbiol., 30: 1989-1994, 1992; Sallsberg et al., J. Med. Virol. 43: 62-68, 1994). In addition, an epitope which genotype-specifically reacts is present in the region from the position 60 to the position 80 of the HCV polypeptide (Machida, Hepatology, 16: 886-891, 1992; Japanese Patent Application 9-209522). Therefore, it is important that an antigen for detection of antibodies against the HCV epitopes has a sequence from the position 1 to the position 40 and from the position 66 to the position 80 of the HCV polypeptide. Accordingly, Example discloses an antigen polypeptide "CEPM" having the sequence from the position 1 to the position 42 and from the position 66 to the position 80 of the HCV polypeptide as an antigen for detection of HCV antibodies. Note that the CEPM is an antigen having an artificial sequence comprising the following regions of the HCV polypeptide in the described order, and a process for construction thereof is described in Japanese Patent Application No. 9-209522. The sequence thereof is described in SEQ ID NO:10.

The linkage of the HCV epitopes of CEPM:

(1238-1312)-(1363-1460)-(1712-1751)-(66-80)-
(1686-1704)-(1716-1751)-(66-80)-(1690-1713)-
(1-42)

On the other hand, for the detection of antigen, monoclonal antibodies recognizing and binding to the region from the position 100 to the position 130 of the HCV polypeptide, antibodies against which region are relatively rare in specimens, is used as a primary antibody, and to detect the core antigen captured by the primary antibody, a monoclonal antibody recognizing a region from the position 40 to the position 50 of the HCV polypeptide, which region is not used for the detection

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JM109, obtaining plasmid pUC·C21-E12.

5 A 1 ng portion of DNA of this plasmid pUC·C21-E12
was subjected to PCR using two primers (5'-
GAATTCATGGGCACGAATCCTAAA-3' (SEQ.ID. No.7), and 5'-
TTAGTCCTCCAGAACCCGGAC-3' (SEQ.ID. No.8)). The PCR was
conducted using a GeneAmp™ Kit (DNA Amplification
Reagent Kit, product of Perkin Elmer Cetus) under
conditions of DNA denaturation at 95°C for 1.5 minutes,
annealing at 50°C for 2 minutes and DNA synthesis at 70°C
10 for 3 minutes, and the resulting DNA fragment was
separated by 0.8% agarose gel electrophoresis and
purified by the glass powder method (Gene Clean).

15 Separately, pUC19 was digested with restriction
enzyme SmaI, and the DNA fragment obtained by PCR was
added to 5 µl of a 10 x ligase buffer solution [660 mM
Tris-HCl (pH 7.5), 66 mM MgCl₂, 100 mM dithiothreitol, 1
mM ATP] and 1 µl of T4 ligase (350 units/µl) with water
to 50 µl, after which overnight incubation was performed
at 16°C for ligation reaction. The plasmid was used to
20 transform *E. coli* JM109, obtaining plasmid pUC19·C21-
E12·SmaI.

25 One microgram of this plasmid DNA was digested at
37°C for one hour with 20 µl of a restriction enzyme
reaction solution [150 mM NaCl, 6 mM Tris-HCl (pH 7.5), 6
mM MgCl₂, 15 units of EcoRI and 15 units of BamHI
enzyme], and this was followed by 0.8% agarose gel
electrophoresis to separate an approximately 490 bp
EcoRI-BamHI fragment, which was then purified by the
glass powder method.

30 A 1 µg portion of DNA of the expression vector
Trp·TrpE (Japanese Unexamined Patent Publication No. 5-
84085) was then digested at 37°C for one hour with 20 µl
of a restriction enzyme reaction solution [150 mM NaCl, 6
mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 15 units of EcoRI and
35 15 units of BamHI enzyme], and after adding 39 µl of
water to the reaction solution and heating it at 70°C for
5 minutes, 1 µl of bacterial alkali phosphatase (BAP)

(250 units/ μ l) was added prior to incubation at 37°C for one hour.

Phenol was added to the reaction solution for phenol extraction, and DNA in the resulting aqueous layer was precipitated with ethanol and dried. A 1 μ g portion of the obtained EcoRI-BamHI treated vector DNA and the aforementioned core fragment were added to 5 μ l of a 10 x ligase buffer solution [660 mM Tris-HCl (pH 7.5), 66 mM MgCl₂, 100 mM dithiothreitol, 1 mM ATP] and 1 μ l of T4 ligase (350 units/ μ l) with water to 50 μ l, and this was followed by overnight incubation at 16°C for ligation reaction.

A 10 μ l portion of this reaction solution was used to transform *E. coli* HB101. A competent *E. coli* strain for the transformation can be prepared by the calcium chloride method [Mandel, M. and Higa, A., J. Mol. Biol., 53, 159-162 (1970)]. The transformed *E. coli* was spreaded onto an LB plate (1% tryptone, 0.5% NaCl, 1.5% agar) containing 25 μ g/ml of ampicillin, and was incubated overnight at 37°C. One loopful of colonies formed on the plate was taken and transferred to LB medium containing 25 μ g/ml of ampicillin for culturing overnight at 37°C.

After centrifuging 1.5 ml of the cell culture solution and collecting the cells, mini-preparation of the plasmid DNA was carried out by the alkali method [Manniatis et al., Molecular Cloning: A Laboratory Manual, 1982)]. A 1 μ g portion of the obtained plasmid DNA was digested at 37°C for one hour with 20 μ l of a restriction enzyme reaction solution [150 mM NaCl, 6 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 15 units of EcoRI and 15 units of BamHI enzyme] and subjected to agarose gel electrophoresis, and a Trp⁺TrpE core 160 expression plasmid producing an approximately 490 bp EcoRI-BamHI fragment was selected.

(B) Expression and purification of polypeptide
encoded by clone core 160

E. coli HB101 bearing expression plasmid Trp·TrpE
core 160 was inoculated in 3 ml of 2YT medium (1.6%
5 tryptone, 1% yeast extract, 0.5% NaCl) containing 50
 $\mu\text{g/ml}$ of ampicillin, and cultured at 37°C for 9 hours.
One milliliter of the culture solution was subcultured at
37°C in 100 ml of M9-CA medium (0.6% Na_2HPO_4 , 0.5% KH_2PO_4 ,
0.5% NaCl, 0.1% NH_4Cl , 0.1 mM CaCl_2 , 2 mM MgSO_4 , 0.5%
10 casamino acid, 0.2% glucose) containing 50 $\mu\text{g/ml}$ of
ampicillin. Indoleacrylic acid was added to a final
concentration of 40 mg/l at the point when $\text{OD}_{600} = 0.3$,
and culturing was continued for 16 hours. The culture
solution was subjected to centrifugation separation to
15 collect the cells.

The cells were suspended by addition of 20 ml of
buffer solution A [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 30
mM NaCl], and further centrifugation yielded 2.6 g of
cells. These obtained cells were suspended in 10 ml of
20 buffer solution A, and after disrupting the *E. coli*
membranes by ultrasonic disruption they were centrifuged
to obtain an insoluble fraction containing a fused
polypeptide of the polypeptide encoded by HCV cDNA and
TrpE. To this fraction there was added 10 ml of buffer
25 solution A containing 6 M urea for solubilization and
extraction of the fused polypeptide. The solubilized
extract was subjected to ion-exchange column
chromatography using S-Sepharose for purification of the
fused polypeptide.

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Example 2. Hybridoma construction method

The fused polypeptide (TrpC11) prepared by the
method described above was dissolved in 6 M urea and then
diluted in a 10 mM phosphate buffer solution (pH 7.3)
35 containing 0.15 M NaCl to a final concentration of 1.0
mg/ml and mixed with an equal amount of TiterMax to make
a TrpC11 suspension. The suspension was adjusted to a

TrpC11 concentration of 0.01 to 0.05 mg/ml and used for intraperitoneal injection into 4- to 6-week-old BALB/c mice. After approximately 8 weeks the immunized animals were further injected through the caudal vein with a physiological saline solution prepared to a TrpC11 concentration of 0.005 to 0.03 mg/ml.

On the third day after the final booster immunization, the spleens of the immunized animals were aseptically extracted, sliced with scissors, broken into individual spleen cells using a mesh, and washed 3 times with RPMI-1640 medium. A logarithmic growth stage mouse myeloma cell line PAI which had been cultured for a few days in the presence of 8-azaguanidine and had the revertants completely removed was washed in the same manner described above, after which 1.8×10^7 of these cells and 1.0×10^8 of the spleen cells were placed in a 50-ml volume centrifuging tube and mixed. This was subjected to centrifugal separation at $200 \times g$ for 5 minutes, the supernatant was removed off, and 1 ml of RPMI-1640 medium containing 50% polyethylene glycol (PEG) 4000 (product of Merck Co.) heated at 37°C was added for cell fusion.

After removing the PEG from the fused cells by centrifugation ($200 \times g$, 5 minutes), a 96-well plate was used for culturing for 1 to 2 weeks in RPMI-1640 medium containing hypoxanthine, aminopterin and thymidine (hereunder abbreviated to "HAT"), to allow growth of only the hybridomas. They were then grown in HAT-free medium, and after about 2 weeks the ELISA method was used to search for clones producing the target antibody, upon which hybridomas were obtained which produced the monoclonal antibody of the invention having the desired reaction specificity.

A conventional limiting dilution procedure was followed to search for and monoclonal the target antibody-producing strains among the obtained hybridomas, and the hybridomas were designated as HC11-14, HC11-10, HC11-3

and HC11-7. These four different hybridomas were deposited at the National Institute of Bioscience and Human Technology on July 4, 1997 as FERM BP-6006, FERM BP-6004, FERM BP-6002 and FERM BP-6003.

Example 3. Preparation of monoclonal antibody

The hybridomas obtained by the method described in Example 2 were transplanted into mice abdomens treated with pristane, etc., and the monoclonal antibodies that were gradually produced in the ascites were collected. The monoclonal antibodies were purified by separating the IgG fraction with a Protein A bound Sepharose column.

The isotype of the monoclonal antibodies produced by the above-mentioned four different hybridomas, C11-14, C11-10, C11-7 and C11-3, were identified by the double immunodiffusion method using each isotype antibody of rabbit anti-mouse IG (product of Zymed Co.), and it was found that C11-10 and C11-7 were IgG2a, and C11-14 and C11-3 were IgG1. As a result of epitope analysis of these four monoclonal antibodies using 20 peptides synthesized by HCV/core region-derived sequences, they were shown to be monoclonal antibodies which specifically recognize portions of the core sequence as shown in Table 1.

Table 1

Antibody	Recognition site
C11-14	⁴¹ Gly- ⁵⁰ Arg (SEQ.ID. No.4)
C11-10	²¹ Asp- ⁴⁰ Arg (SEQ.ID. No.3)
C11-3	¹⁰⁰ Pro- ¹²⁰ Gly (SEQ.ID. No.5)
C11-7	¹¹¹ Asp- ¹³⁰ Phe (SEQ.ID. No.6)

Example 4. Method for efficient detection of antigen without pre-processing procedure

A specimen containing HCV particles was diluted in a containing detergents reaction solution to determine the efficiency with which HCV core antigen was detected.

The detection of the HCV core antigen was performed by the sandwich enzyme immunoassay (EIA) using monoclonal antibodies for the HCV core antigen. Of the monoclonal antibodies obtained in Example 3, C11-3 and C11-7 were used as antibodies for capturing the core antigen, and C11-10 and C11-14 were used as antibodies for detection of the captured core antigen.

The EIA was performed basically under the following conditions. Solutions of monoclonal antibodies C11-3 and C11-7 in acetate buffer solutions diluted to 4 $\mu\text{g/ml}$ each were added to microtiter plates and incubated overnight at 4°C. They were then washed with a phosphate buffer solution and subjected to a blocking procedure by addition of phosphate buffer solution containing 1% BSA. After adding 100 μl of the reaction solution and 100 μl of the specimen thereto and mixing, the reaction was carried out at room temperature for 1.5 hours. After removing the unreacted materials by washing with a phosphate buffer solution containing a low concentration of detergent, monoclonal antibodies C11-10 and C11-14 labeled with alkali phosphatase were added for reaction at room temperature for 30 minutes. After completion of the reaction, the unreacted materials was removed by washing with a phosphate buffer solution containing a low concentration of detergents, and a substrate solution (CDP-Star/emerald11) was added for reaction at room temperature for 15 minutes, after which the luminescence was measured.

Different detergents were added to the primary reaction solution and their effects were examined. HCV antigen-positive serum with an anti-HCV antibody titer below the detection level and thought to contain virtually no anti-HCV antibody was used, and the core antigen detection sensitivities were determined based on the degree of luminescence and expressed as reaction ratios relative to 1.0 as the luminescence for healthy human serum. The results are shown in the following

Tables 2 and 3.

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Table 2

		Reaction ratio (S/N ratio) for each serum with respect to healthy human serum				
		No.45	No.46	No.3	No.7	No.19
Not added		15.67	1.00	1.15	1.34	1.19
Effect judgment criterion		>30.0	>2.0	>2.0	>2.0	>2.0
		HLB value				
		%				
Anionic detergent	sodium dodecyl sulfate	40.0	0.5	5.42		
			2.0	5.73		
	sodium dodecyl-N-sarcosinate		0.5	12.79	2.70	
Cationic detergent			2.0	125.43	7.27	3.83
	perfluoroalkylcarboxylic acid S-113 (ASAHI GLASS)		0.5	10.55	1.27	
			2.0	6.72	0.91	
	cetyltrimethylammonium bromide		0.5	72.97	7.42	3.09
			2.0	44.55	5.35	
	dodecylpyridinium chloride		0.5	53.43	4.70	2.05
			2.0	12.44	2.49	
	n-dodecyltrimethylammonium		0.5	66.84	4.43	2.41
			2.0	27.98	3.77	
	tetradecylammonium bromide		0.05	14.69		
			0.5	12.57		
	n-octyltrimethylammonium chloride		2.0	11.46	1.00	0.75
Non-ionic detergent	CHAPS		0.5	29.57		
			2.0	25.32	1.63	1.82
	perfluoroalkylbetaine S-132 (ASAHI GLASS)		0.5	11.07	1.61	
			2.0	10.77	1.49	
	3-(dodecyltrimethylammonio)-1-propanesulfonic acid		0.5	57.69		
			2.0	113.19	4.57	3.44
						5.26

Table 3

		Reaction ratio (S/N ratio) for each serum with respect to healthy human serum				
Not added		No.45	No.46	No.3	No.7	No.19
Effect judgment criterion		15.67	1.00	1.15	1.34	1.19
		>30.0	>2.0	>2.0	>2.0	>2.0
Additive		HLB value				
		%				
Non-ionic detergent	MEGA-10	0.5	32.11	3.38		
		2.0	38.49	3.53	1.97	1.87 2.84
	Tween 20	0.5	16.88			
		2.0	12.36			
	Tween 40	0.5	14.96		1.02	0.99 1.41
		2.0	19.10		1.32	1.25 1.64
	Tween 80	0.5	12.45		1.33	1.23 1.10
		2.0	17.47			
	Nonidet P-40	0.5	43.14		3.09	2.95 4.58
		0.5	12.48		0.90	0.60 0.97
	octyl glycoside	2.0	25.07		1.92	1.20 2.63
		0.5	26.50		1.85	1.62 2.70
	Triton N101	2.0	60.84		2.23	2.28 3.81
		0.5	27.72			
	Triton X100	2.0	71.08		2.90	2.34 3.86
		0.5	31.49		2.04	1.65 2.77
	Triton X114	2.0	58.62		1.92	2.11 2.51
		0.5	10.50		0.94	0.97 1.08
	Triton X305	2.0	25.91		1.30	1.24 1.87
		0.5	12.54		0.86	0.78 1.04
	Triton X405	2.0	24.92		1.21	1.24 1.25
		0.5	5.45	1.00		
Others	benzyltrimethylphenylammonium chloride	2.0	7.01	1.12		
		0.5	3.89	0.97		
Detergent mixture	triethylamine					
	2% sodium dodecyl-N-sarcosinate + 2% Triton X100		244.13		6.11	5.50 12.71

These results demonstrated that addition of non-ionic detergents exhibiting HLB values of 12 to 14, as represented by Triton X100, increased the luminescence and raised the detection sensitivity in HCV antigen-positive serum with respect to healthy human serum. It was also demonstrated that addition of detergents with both linear alkyl chains and secondary to quaternary amines in their structures, as represented by sodium dodecyl-N-sarcosinate and dodecyltrimethylammonium, also raised the detection sensitivity in HCV antigen-positive serum. No such sensitivity-raising effect was found with the surfactants having alkyl groups of 8 or fewer carbon atoms. Addition of a mixture of these two types of surfactants (mixture of 2% sodium dodecyl-N-sarcosinate + 2% Triton X100 in Table 2) was also shown to raise the detection sensitivity in HCV antigen-positive serum.

Example 5. Core antigen detection in specimens after HCV infection and before HCV antibody appearance (during window period)

A commercially available PHV905 seroconversion panel (BBI Inc.) was measured according to Example 4 with addition of 2% Triton X100 and 2% sodium dodecyl-N-sarcosinate to the reaction solution. The PHV905 panel used here exhibited positive conversion in the anti-HCV antibody test (Ortho EIA. 3.0) on the 21st day after initial observation (Serum No. PHV905-7), the antibody titer being represented by the cutoff index (S/CO), with 1.0 or greater judged as positive. The HCV core antigen activity (luminescence) was represented as a ratio (S/N) with respect to 1.0 as the luminescence for healthy human serum.

As shown in Table 4, core antigen activity was found even before anti-HCV antibody positivity, thus confirming that addition of the surfactant resulted in exposure of the core antigen from the viral particles, thus allowing its reaction with the immobilized monoclonal antibodies

and its detection.

Table 4

Serum No.	Days after initial observation	HCV core antigen activity (S/N)	Anti-HCV antibody titer (S/CO)
PHV 905-1	0	5.32	0.000
905-2	4	8.30	0.000
905-3	7	15.63	0.000
905-4	11	4.37	0.300
905-5	14	14.75	0.700
905-6	18	7.57	0.700
905-7	21	4.82	2.500
905-8	25	3.31	5.000
905-9	28	1.61	5.000

Example 6. Simultaneous detection of HCV antibody and core antigen in specimen

A specimen (human serum) was used which contained an antibody for an HCV epitope but virtually no HCV antigen, and the following method was used to confirm that it was possible for the antibody for the HCV epitope to bind with the HCV polypeptide in the primary reaction solution containing the detergent without inactivation and for it to be detected by addition of anti-human antibody in a secondary reaction solution, and to confirm that it was possible to detect the core antigen when the core antigen was present, the antibody when the antibody for the HCV epitope was present, and both when both were present.

The EIA was performed basically under the following conditions. Recombinant antigen CEPN containing an HCV epitope was diluted in a urea-containing phosphate buffer solution, and then added to a microtiter plate and incubated overnight at 4°C. After washing with a phosphate buffer solution, a solution prepared by diluting monoclonal antibodies C11-3 and C11-7 in an

acetate buffer solution was added to the plate and incubated overnight at 4°C. The method of preparing the recombinant antigen CEPm is described in Japanese Patent Application No. 9-209522. After removal of the antibody solution, it was washed with a phosphate buffer solution and subjected to a blocking procedure by addition of a phosphate buffer solution containing 1% BSA.

After adding 100 µl of a primary reaction solution containing Triton X100, sodium dodecyl-N-sarcosinate and urea and 100 µl of the specimen in that order and stirring, the reaction was carried out at room temperature for 1.5 hours. After removing the unreacted matter by washing with a phosphate buffer solution containing a low concentration of added surfactant, a secondary reaction solution containing the anti-HCV core antigen monoclonal antibody C11-14 labeled with horseradish peroxidase and anti-human IgG mouse monoclonal antibody was added for reaction at room temperature for 30 minutes.

After completion of the reaction, the unreacted matter was removed by washing with a phosphate buffer solution containing a low concentration of added surfactant, and a substrate solution (ortho-phenylenediamine) was added for reaction at room temperature for 20 minutes, after which the luminescence was measured.

HCV antibody-positive human serum which had been confirmed to contain virtually no HCV core antigen was diluted with horse serum and used for specimens to confirm detection of antibodies for the HCV epitope, by which it was found to react in a concentration-dependent manner and the antibody was confirmed to be detected without inactivation in the primary reaction solution.

Table 5 Simultaneous measurement of HCV antigen and HCV antibody

Labeled antibody:		(Comp. Ex.)	(Comp. Ex.)	(Invention)
		POD-labeled c11-14	POD-labeled anti-human IgG	POD-labeled c11-14 and POD-labeled anti-human IgG
Solid phase		c11-3 and c11-7	CEPM	c11-3 and c11-7 and CEPM
Sample				
Recombinant core antigen ng/ml	Positive serum dilution degree			
-	-	0.001	0.000	0.000
50	-	2.784	0.000	2.834
12.5	-	2.822	0.000	2.758
3.1	x2048	1.586	0.210	1.341
0.78	x512	0.423	0.539	0.815
0.2	x128	0.085	1.139	1.151
0.048	x32	0.014	1.746	1.621
-	x8	0.000	2.161	1.824

(Values: OD492/OD690)

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Separately, a recombinant core antigen was added to horse serum and diluted with horse serum for use as specimens, which were measured to confirm that the recombinant core antigen could be detected in a concentration-dependent manner.

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When specimens containing appropriate amounts of added core antigen and human serum were used as specimens, a signal for the recombinant core antigen was obtained when only the recombinant core antigen was present, a signal for only the HCV antibody was obtained when only the human HCV antibody-positive serum was present, and a summated signal of both signals was obtained when both were present, as shown in Table 4. It was thus shown that both the antigen detection system and the antibody detection system functioned properly without interfering with each other, thus allowing detection of the HCV antigen and the antibody for the HCV polypeptide epitope.

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Example 7. Antigen-antibody assay method for human serum

A healthy human specimen, a patient specimen and a serum positive-conversion panel specimen (BBI, Inc.) were used for simultaneous measurement of antigen and antibody according to the method described in Example 6. The panel serum was used for comparison with the results determined using a vending agency-supplied HCV antibody detection reagent.

The results of measurement of 18 healthy human specimens are shown in Table 6, and confirm that no reaction occurred in the healthy persons. Based on the distribution for the healthy persons, the critical value between positivity and negativity was determined to be 0.1.

Positive values were exhibited for all of the HCV-positive specimens, as shown in Table 7.

However, as shown in Table 8, the points 1 to 6 for which positivity could not be determined in antibody testing of the panel sera were given positive judgments. These points were given positive judgments in the results of the Amplicor HCV test, and correspond to the window period, and therefore positivity was also confirmed with the window period specimens.

Table 6

Specimen No.		Absorption
Healthy human	1	0.063
	2	0.057
	3	0.066
	4	0.025
	5	0.045
	6	0.063
	7	0.047
	10	0.033
	11	0.036
	13	0.037
	14	0.030
	15	0.028
	16	0.031
	17	0.040
	18	0.051
	19	0.052
	20	0.031
	21	0.053
mean		0.044

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Table 7

Patient specimens	Absorption
3	2.892 positive
16	2.335 positive
45	0.394 positive
84	2.769 positive

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Table 8

Panel sera	Absorption	Judgment	Antibody assay	Amplicor HCV test
PHV907-1	0.557	positive	negative	positive
2	0.397	positive	negative	positive
3	0.357	positive	negative	positive
4	0.224	positive	negative	positive
5	0.192	positive	negative	positive
6	0.247	positive	positive	positive
7	2.414	positive	positive	positive

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obtained.

Example 10. Sandwich ELISA using different immobilized monoclonal antibodies

Monoclonal antibody (C11-3, C11-5, and C11-15; C11-3
5 and C11-7; C11-3 and C11-15; C11-3 alone; C11-7 alone; or
C11-15 alone) was diluted in 10 mM phosphate buffer (pH
7.3) to a final concentration of 6 μ g/ml, and 100 μ l of
the solution was added to each well of microplate. After
allowing to stand at 4°C overnight, the buffer was
10 aspirated off, and the wells were washed twice with 10 mM
phosphate buffer (pH 7.3). 350 μ l of 10 mM phosphate
buffer (pH 7.3) containing 0.5% casein was added, and
after incubation at room temperature for two hours, the
buffer was aspirated off. 100 μ l of a specimen which is
15 HCV-RNA positive and anti-HCV antibody negative and 100
 μ l of a reaction solution were added to each well, and
reacted at room temperature for an hour. After washing,
a peroxidase-labeled anti-core antigen monoclonal
antibody (a mixture of C11-14 and C11-10) was added, and
20 reacted for 30 minutes, and after washing, an enzyme
reaction was carried out by adding a substrate solution
comprising orthophenylenediamine and hydrogen peroxide.
After reaction at room temperature for 30 minutes, 2N
sulfuric acid was added to terminate the enzyme reaction,
25 and the absorbance at 292 nm was measured by a microplate
reader. A result is shown in Fig. 2.

It was revealed that although detection sensitivity
is low if only C11-15 was immobilized, the detection
sensitivity increases by monobilizing a mixture of C11-
30 15, and C11-3 or C11-7 etc.

Example 11. Expression and purification of epitope-chimeric antigen

E. coli transformant CEPM/HB101 was cultured in an
LB medium containing 100 μ g/ml ampicillin at 37°C
35 overnight. The culture was inoculated to M9-CA
containing 100 μ g/ml ampicillin by an inoculum size of 1%
concentration, and cultured at 37°C overnight. After

finishing the culturing, the microbial cells were collected by centrifugation, and resuspended in 50 ml of a lysis solution [50 mM Tris-HCl (pH 8.5), 30 mM NaCl, 5 mM EDTA), and after adding 1 ml of a lysozyme solution (10 mg/ml Lysozyme), were inoculated at 37°C for an hour. This suspension was subjected to ultrasonication (150W, 90 seconds, twice) so as to disrupt the cells. An insoluble fraction was recovered by centrifugation at 15000 rpm, 4°C for 30 minutes. The insoluble fraction was resuspended in 50 ml of A solution [50 mM Tris-HCl (pH 8.5)] containing 1% NP40, and homogenated (1500 rpm, 5 strokes). The suspension was centrifuged at 1500 rpm, 4°C for 30 minutes to recover an insoluble fraction. The insoluble fraction was resuspended in 50 ml of the A solution containing 2M urea, and homogenized (1500 rpm, 5 strokes). The suspension was centrifuged at 15000 rpm, 4°C for 30 minutes to recover an insoluble fraction. The insoluble fraction was resuspended in 50 ml of the solution A containing 6M urea, and homogenized (1500 rpm, 5 strokes). The suspension was centrifuged at 15000 rpm, 4°C for 30 minutes to recover a soluble fraction.

The epitope chimeric antigen was purified from the antigen solution prepared by solubilization with 6M urea-containing solution, by an ion exchange using S Sepharose HP column (Pharmacia) and gel-filtration using Superdex 75 pg (Pharmacia).

Note that an nucleotide sequence of DNA encoding said chimeric antigen is shown in SEQ ID NO:10, and the amino acid sequence of the chimeric antigen is shown in SEQ ID NO:11.

Reference to Microorganisms deposited under Rule 13-2 of PCT and Depository Institute
Depository Institute

Name: The National Institute of Bioscience and Human Technology

Address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan

(1) Identification: HC11-3

Deposition date: July 4, 1997

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(2) Identification: HC11-7

Deposition date: July 4, 1997

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(3) Identification: HC11-10

Deposition No: FERM BP-6004

Deposition date: July 4, 1997

(4) Identification: HC11-11

Deposition No: FERM BP-6005

Deposition date: July 4, 1997

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(5) Identification: HC11-4

Deposition No: FERM BP-6006

Deposition date: July 4, 1997

(6) Identification: HC11-15

Deposition No: FERM BP-6782

Deposition date: July 16, 1999

Ans. c. 17